Gene Therapy for Hemophilia B: Host Immunosuppression Prolongs the Therapeutic Effect of Adenovirus-Mediated Factor IX Expression

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ABSTRACT

Hemophilia B is caused by a deficiency of blood clotting factor IX (FIX). Previous studies have shown that the delivery of a recombinant adenoviral vector expressing canine FIX (cFIX) resulted in a complete correction of hemophilia B in FIX-deficient dogs, but that cFIX expression decreased to only about 1–2% of normal levels 3 weeks after treatment. In the present study, therapeutic levels of cFIX expression capable of producing a partial correction of hemophilia B were maintained for at least 6 months after the coadministration of the cFIX-expressing adenovirus and the immunosuppressive agent cyclosporin A (CsA). These findings support a recent report (Yang et al., 1994) that host T-cell-mediated immunity against virally transduced cells is a major contributing factor to the transient nature of adenovirus-mediated gene expression in immunocompetent animals. Although a second administration of the cFIX-expressing adenovirus 6 months after the first infusion had only a minimal effect on plasma FIX levels in a dog that had been continuously treated with CsA, the prolonged expression of the transgene indicates that immunosuppression may be applicable in attaining long-term treatment of clinically relevant disorders.

OVERVIEW SUMMARY

Adenovirus-mediated gene transfer in vivo results in efficient but transient transgene expression. Recent studies have suggested that this loss of expression is caused by the destruction of adenovirally transduced cells by the host immune system. If true, suppression of the immune system should significantly prolong the effects of adenoviral treatment. This hypothesis was examined by combining adenovirus-mediated transfer of the canine factor IX cDNA with continuous immunosuppression in a dog model for hemophilia B. Immunosuppression significantly increased the persistence of transgene expression following adenovirus-mediated transfer in vivo. This result suggests that further modifications of adenoviral vectors that reduce their immunogenicity may significantly increase their persistence in vivo.

INTRODUCTION

Hemophilia B is caused by a hereditary defect in blood clotting factor IX (FIX). The incidence of this disease is about 1 in 30,000 males, representing about 20% of all hemophiliacs (Roberts and Lozier, 1991). Affected individuals require regular administration of FIX concentrates prepared from human plasma to prevent or stop bleeding episodes. Although the complications resulting from contamination with blood-borne pathogens, such as human immunodeficiency and hepatitis B viruses, can be reduced through the use of recombinant FIX, severely affected patients are still at risk for life-threatening hemorrhage and other complications from repeated bleeding episodes due to the relatively short half-life of FIX in the circulation. Continuous supply of this coagulating factor is therefore preferable to intermittent administration of concentrates. Current research efforts are focusing on the development of
of novel therapies for hemophilia B based on the delivery of the FIX gene or cDNA into appropriate target cells, so that sufficient amounts of FIX will be constantly produced to alleviate or cure the disease (Dai et al., 1992; Kay et al., 1993, 1994; Smith et al., 1993; Lozier and Brinkhous, 1994; Yao et al., 1994).

Previous studies have shown that infusion of a retroviral vector containing the cFIX cDNA into the portal vasculature could directly transduce hepatocytes of the hemophilia B dog in vivo, resulting in constitutive expression of low levels of cFIX for more than 11 months (Kay et al., 1993; Lozier and Brinkhous, 1994). Reductions of whole blood clotting and partial thromboplastin times were also observed in the treated animals. However, retrovirus-mediated in vivo gene delivery is extremely invasive, necessitating the removal of 70% of the liver from the individuals. Levels of transgene expression are also too low to have a therapeutic effect in many cases. Low levels of expression were also observed with ex vivo gene transfer approaches (Dai et al., 1992; Yao et al., 1994). In contrast, in vivo gene delivery using recombinant adenoviral vectors has been shown to be highly efficient (Smith et al., 1993; Kay et al., 1994). At relatively safe adenoviral doses, supranormal levels of plasma FIX could be achieved in hemophilia B dogs 1–3 days after infusion of a recombinant adenoviral vector containing the cFIX cDNA, resulting in a complete phenotypic correction of the hemophilic state (Kay et al., 1994). Because adenoviral vectors can transduce quiescent cells, partial hepatectomy is not required in adenovirus-mediated gene therapy. Unfortunately, initial studies of adenovirus-mediated gene therapy showed that high levels of transgene expression were only transient. FIX levels in treated hemophilia B dogs declined by about 3 log units during the first 3 weeks of treatment, and slowly decreased to undetectable levels by 6 months after treatment (Kay et al., 1994). More recent studies have suggested that this loss of transgene expression is largely due to a host immune response directed against the cells transduced by the recombinant adenovirus (Yang et al., 1994). Because long-term expression of a transgene at therapeutic levels is necessary for the treatment of most genetic defects, including hemophilia B, we have examined whether the coadministration of the cFIX-expressing adenoviral vector and the immunosuppressive agent cyclosporin A (CsA) could significantly prolong the expression of therapeutic levels of FIX in hemophilia B dogs.

All viral preparations were proven to be free of endotoxin by assays with a third-generation pyrogen testing kit from BioWhittaker (Catalog No. 50-648U).

Animal studies

Hemophilia B dogs completely deficient in FIX were maintained at the Francis Owen Blood Research Laboratory, University of North Carolina at Chapel Hill. All animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23). Experiments for non-cyclosporin A-treated dogs have been reported (Kay et al., 1994). Adenoviral infusion into cyclosporin A-treated dogs was performed via a catheter to the cephalic vein. Cyclosporin A was administrated to the dogs orally at a dosage of 300 mg/dog-day. This is equivalent to an average dose of 19.5 mg/kg. The optimal dose of cyclosporin in the dog has been reported as 18 mg/kg (Hasegawa et al., 1992). The first dose of cyclosporin was given prior to or immediately after vector infusion (Schrieber and Crabtree, 1992).

Hemostatic analysis

Whole blood clotting times (WBCTs) and FIX levels were determined as previously described (Langdell et al., 1989, 1993). The ELISA immunoassay for quantitation of FIX antigen was performed using a polyclonal rabbit anti-dog FIX antibody as previously described (Kay et al., 1994).

Cyclosporin assay

Whole blood CsA values were determined as described (Wang et al., 1990).

Plaque neutralization assay

The plaque neutralization assay was performed according to the method described by Pacini et al. (1984).

RESULTS

FIX levels in hemophilia B dogs treated with the adenoviral vector Ad.RSV-cFIX and CsA

The adenoviral vector used in the present study, Ad.RSV-cFIX, is the same as that reported previously (Kay et al., 1994). Before infusion into dogs, all viral preparations were tested for function and toxicity in vivo by infusion of 1010 purified viral particles into the tail vein of BALB/C mice. Serum cFIX antigen levels determined by ELISA were similar to those observed in previous studies (data not shown). Endotoxin assays were negative.

As reported previously (Kay et al., 1994), infusion of 1.6 × 1011 pfu/kg of Ad.RSV-cFIX into the splenic veins of two hemophilia B dogs resulted in 250–300% of normal plasma FIX levels at 1–2 days post-treatment (Fig. 1). Plasma FIX levels in these 2 animals rapidly decreased to about 1% of normal levels at 3 weeks after infusion, and then slowly decreased to less than 0.1% of normal levels by 2 months. In the present experiment, 2 hemophilic animals were infused via the cephalic vein

MATERIALS AND METHODS

Construction of an adenoviral vector expressing canine factor IX

The construction of the adenoviral vector containing the cFIX cDNA under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter has been described (Kay et al., 1994). The recombinant adenovirus was purified by two cycles of CsCl banding and dialyzed extensively with 10 mM Tris HCl pH 7.4, 1 mM MgCl2, and 10% glycerol. The purified virus was stored at −80°C. The virus titer, as determined by OD260, ranged from 1 to 3 × 1011 pfu/ml. Titters measured by plaque assay (Graham and Prevec, 1991) were usually within one order of magnitude of the OD260 titters.
with the cFIX-expressing adenoviral vector Ad.RSV-cFIX and the immunosuppressant CsA. One dog, which received $2.2 \times 10^{11}$ pfu/kg, attained a peak level of plasma FIX of 480% of normal at day 2; FIX levels were maintained at 108% of normal through day 21 (Fig. 1). Plasma FIX levels were about 10% of normal at 2 months and about 2% of normal at 6 months after treatment. Thus, in this 1 animal, cyclosporin treatment largely prevented the rapid decrease in factor IX expression observed during the first 3-week period in the original experiment. To confirm this result, a second dog was treated with CsA but received a lower viral dose ($1 \times 10^{11}$ pfu/kg). Although the peak level was lower than in the previous animal, reflecting the lower viral dose administered, a similar pattern of persistence was observed (Fig. 1).

**Whole blood clotting time in hemophilia B dogs treated with Ad.RSV-cFIX and CsA**

Before administration of Ad.RSV-cFIX, whole blood clotting times (WBCTs) in all of the hemophilia B dogs were greater than 50 min. Within 1–8 days after infusion of $1.6 \times 10^{11}$ pfu/kg of Ad.RSV-cFIX alone, WBCTs became normal or near normal but increased slowly to over 20 min at the end of 3 months (Fig. 2). These changes in WBCT were consistent with changes in plasma FIX levels. Although the differences in WBCT between cyclosporin A-treated and non-cyclosporin A-treated animals were not statistically significant due to high variability in control animals and low number of animals examined, administration of cyclosporin A maintained near normal WBCTs for up to 10 months (Fig. 2).

**CsA trough levels in treated animals**

Trough levels of CsA in whole blood of dogs receiving immunosuppression were monitored periodically by fluorescence polarization assays. The cyclosporin A trough levels were usually in the range of 300–600 ng/ml, but were always between 100 ng/ml and 1100 ng/ml (data not shown).

**Anti-adenovirus antibodies in cyclosporin A-treated dogs**

Sera from CsA-treated dogs were periodically monitored for anti-adenovirus antibodies by plaque neutralization assay. High levels of anti-adenovirus antibodies were detected in both dogs 1 week after viral infusion (Fig. 3). After achieving peak levels of 1:1,280 at 3 weeks, serum anti-adenovirus antibodies were maintained at 1:480 to 1:160 for at least 6 months.

**Repeated administration of Ad.RSV-cFIX in CsA-treated hemophilia B dogs**

To determine whether the gradual decrease in serum levels of neutralizing antibodies would permit additional cFIX expression from the recombinant adenovirus, 1 dog was given a second dose of Ad.RSV-cFIX, and plasma FIX levels and WBCTs were monitored before and after this second infusion.
The viral dose for the second infusion was $1.34 \times 10^{11}$ pfu/kg, for a total of $2.1 \times 10^{12}$ pfu. Changes in plasma FIX levels after the second infusion were minimal (Fig. 1). A slight increase in plasma FIX from 1.8% to 3.1–3.4% of normal was observed at 1–2 days after infusion. Changes in WBCTs were also minimal (Fig. 2). This minimal change in WBCT was anticipated, because WBCT in this dog was still in near normal range before the second administration. Antibody levels were increased dramatically by the reinfusion of the adenoviral vector (Fig. 3).

**DISCUSSION**

The roles of host cellular immune response to human adenovirus 5 have been evaluated in mice by Müllbacher et al. (1989). The cytotoxic T lymphocyte response to Ad5 was class I major histocompatibility complex (MHC)-restricted and mapped to the k end of MHC in CBA (H-2k) mice. Experiments with different viral mutants suggested that the E1 and E2 regions of the Ad5 genome are important for target cell sensitivity to lysis by Ad5-immune T cells. Target cell lysis was much reduced, but still detectable in cells infected with E1A- or E1B-deleted Ad5 virus (Müllbacher et al., 1989). More recently, cellular immunity to an E1-deleted recombinant adenoviral vector has also been reported (Engelhardt et al., 1994; Yang et al., 1994). Following instillation of a recombinant adenovirus encoding lacZ into immunocompetent (CBA) and genetically athymic (nu/nu) mouse strains, transgene expression diminished from >80% of hepatocytes at day 2 to undetectable levels by day 21 in CBA mice, but was maintained essentially unchanged for 60 days in nu/nu mice. Progressive loss of viral DNA was detected in CBA mice but not in nu/nu mice (Yang et al., 1994). Following treatment of immunocompetent mice with CsA, lacZ transgene expression was extended to 21 days as compared with 14 days in untreated animals (Engelhardt et al., 1994). In the present study, the therapeutic effects of adenovirus-mediated gene therapy could be extended for nearly 6 months in adult hemophilia B dogs by the administration of CsA. Thus, a combined therapy of adenovirus-mediated gene transfer and immunosuppression might be applicable in some clinical cases, especially where treatment of a life-threatening disorders require long-term high-level transgene expression.

Cytotoxic T lymphocytes are evoked by class I MHC-restricted antigens, which are synthesized within cells and presented together with class I MHC on the cell surface. Because E1-deleted adenovirus can still replicate without E1 complementation in trans in cells infected at high multiplicity (Shenk et al., 1980), viral protein expression from the E1-deleted adenoviral genome in transduced cells is not unexpected. In fact, Ad5 DNA-binding protein and hexon protein were detected in the livers of the mice infused with E1-deleted adenoviral vector (Yang et al., 1994). Provoked cytotoxic T lymphocytes will destroy transduced cells, leading to the elimination of transgene and thus limiting the therapeutic effect of adenoviral-mediated gene therapy to a short period of time. By blocking host cellular immune response with an immunosuppressive agent, the genetically modified cells can elude host immune surveillance, resulting in prolonged expression of the transgene and the therapeutic effect of adenovirus-mediated gene transfer. This hypothesis was tested and supported by the present studies in hemophilia B dogs. Cyclosporin A, a fungal metabolite, exhibits strong immunosuppressive properties and is widely used.

**FIG. 2.** Whole blood clotting times (WBCTs) in hemophilia B dogs after treatment with Ad.RSV-cFIX in the presence or absence of cyclosporin A. The experimental design is identical to that described in Fig. 1.
as an immunomodulator for combating allograft rejection due to its low myelotoxicity and specificity for T lymphocytes (Borel et al., 1977; Shevach, 1985). The results from the present study showed that CsA can be used to extend the effects of adenovirus-mediated gene therapy. Although the mechanisms of CsA-mediated immunosuppression are not fully understood, it is known that CsA can inhibit early events in T cell activation, such as activation of interleukin-2 gene expression (Borel and Gunn, 1986; Heitman et al., 1993). CsA is also reportedly able to inhibit transcription from the adenovirus major late promoter (Mahajan and Thompson, 1993). It is unclear if this function of CsA is also involved in its effects on adenovirus-mediated gene therapy in the hemophilia B dogs. Nevertheless, in the present studies, host humoral immune response was not suppressed, since a high level of adenovirus neutralization antibodies was detected in the CsA-treated dogs and repeated administration of adenoviral vector in 1 dog 6 months after the first infusion showed only minimal effects on plasma FIX levels.

A slower decline of plasma FIX levels over time was observed in CsA-treated dogs. It is unclear if this decline was due to unsuppressed host immune function from residual cytotoxic T lymphocytes and/or antibody-dependent cellular cytotoxicity (ADCC), or due to “natural” loss of the nonintegrated adenoviral gene in transduced cells. This gradual decline of plasma FIX levels occurred even though the CsA dose was optimal, and no antibodies against canine factor IX were detected in these animals (data not shown). Not surprisingly, rejection of allografts has previously been reported under such optimal dosing (Hasegawa et al., 1992; Fujisawa et al., 1993). Because the adenoviral genome is not integrated into the host cellular genome, degradation of the adenoviral genome within transduced cells or loss of the transduced cell through the natural process of cell turnover may also have resulted in reduction of transgene expression in the target tissues or organs. A better understanding of the mechanisms underlying this slow decline of transgene expression will be important for the future design of adenoviral vectors aimed at long-term gene therapy.

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FIG. 3. Anti-adenovirus neutralizing antibodies in the 2 hemophilia B dogs treated with Ad.RSV-cFIX and cyclosporin A. The solid arrow indicates the point at which 1 dog was reinfused with $1.34 \times 10^{11}$ pfu of Ad.RSV-cFIX/kg.


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